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Delineation of a Conserved B Cell Epitope on Bonnet Monkey (*Macaca radiata*) and Human Zona Pellucida Glycoprotein-B by Monoclonal Antibodies Demonstrating Inhibition of Sperm-Egg Binding[1]

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ABSTRACT

To circumvent autoimmune oophoritis after immunization with zona pellucida (ZP) glycoproteins, synthetic peptides encompassing B cell epitope(s) and devoid of oophoritogenic T cell epitopes as immunogens have been proposed. In this study, bonnet monkey (*Macaca radiata*) ZP glycoprotein-B (bmZPB) was expressed as polyhistidine fusion protein in *Escherichia coli.* Rabbit polyclonal antibodies against recombinant bmZPB (r-bmZPB) significantly inhibited human sperm-oocyte binding. To map B cell epitopes on ZPB, a panel of 7 murine monoclonal antibodies (mAbs) was generated against r-bmZPB. All 7 mAbs, when tested in an indirect immunofluorescence assay, reacted with bonnet monkey ZP, and only 6 recognized human zonae. Monoclonal antibodies MA-809, -811, -813, and -825 showed significant inhibition in the binding of human spermatozoa to human ZP in a hemizona assay. Epitope-mapping studies using multipin peptide synthesis strategy revealed that these 4 mAbs recognized a common epitope corresponding to amino acids (aa) 136–147 (DAPDTDWCDSIP). Competitive binding studies revealed that the synthetic peptide corresponding to the identified epitope (aa 136–147) inhibited the binding of MA-809, -811, -813, and -825 to r-bmZPB in an ELISA and to bonnet monkey ZP in an indirect immunofluorescence assay. The epitopic domain corresponding to aa 136–147 of bmZPB was completely conserved in human ZPB. These studies will further help in designing ZP-based synthetic peptide immunogens incorporating relevant B cell epitope for fertility regulation in humans.

INTRODUCTION

The mammalian oocyte is surrounded by an acellular translucent envelope termed the zona pellucida (ZP). It is composed of three distinct glycoproteins, which on the basis of the size of mRNA transcripts have been classified as ZPA, ZPB, and ZPC [1]. ZP glycoproteins serve as the docking site for species-specific sperm binding, induce bound spermatozoa to undergo the acrosome reaction, and affect block to polyspermy [2]. In the mouse model, during fertilization, spermatozoa initially bind to ZPC, which triggers the acrosome reaction [3]. Subsequent to the acrosome reaction, ZPA acts as the secondary receptor and helps in the maintenance of acrosome-reacted spermatozoa binding to the oocyte [4]. It has been postulated that ZPB crosslinks the ZPA-ZPC heterodimer. However, in other species such as the pig, recent observations suggest that ZPC associates with ZPB, leading to high molecular weight heterocomplexes that bind with very high affinity to boar sperm-associated zona receptors, thereby suggesting that both the glycoproteins participate in the sperm binding process [5]. The critical involvement of ZP glycoproteins in reproduction, together with their tissue-specific nature, has made these promising target antigens for development of an immunocontraceptive vaccine. Immunization of female subjects with ZP glycoproteins leads to block of fertility, which is invariably associated with either transient or irreversible alteration in the cyclicity, hormonal profile, and follicular development in the ovary [6–9]. The presence of oophoritogenic T cell epitopes in the immunogen has also been implicated as one of the factors responsible for this phenomenon [10]. Segregation of the oophoritogenic T cell epitope(s) and immunization of female mice with a chimeric peptide comprising a promiscuous foreign T cell epitope and modified peptide corresponding to B cell epitope of mouse ZPC leads to block in fertility without concomitant autoimmune oophoritis [11].

Recently, we have cloned bonnet monkey (*Macaca radiata*) ZPB (bmZPB) and revealed that its deduced amino acid (aa) sequence has 92.0% sequence identity with human (h) hZPB [12]. In cynomolgus monkeys, ZPB transcript is present in oocytes in secondary follicles and to a lesser extent in tertiary follicles, but it is absent in primordial, primary, or antral follicles and in granulosa cells [13]. However, ZPA and ZPC transcripts are present not only in oocytes at all stages of folliculogenesis but also in granulosa cells [13]. Absence of ZPB transcript from primordial and primary oocytes suggests that antibodies against the ZPB might not affect the resting pool of primordial follicles in the ovary. The ovarian damage can further be eliminated by use of synthetic peptide(s) constructed to contain the bioeffective B cell epitope segregated from the oophoritogenic T cell epitope.

In the present study, we have developed monoclonal antibodies (mAbs) against recombinant (r) bmZPB, expressed in *E. coli* as a polyhistidine fusion protein, excluding N-terminal signal sequence and C-terminal transmembrane-like domain. Using multipin peptide synthesis strategy, the epitopes recognized by mAbs, including those capable of inhibiting in vitro binding of human sperm with human zonae in a hemizona assay, have been mapped. Moreover, the epitope recognized by bioeffective mAbs is found to be well conserved with hZPB. These studies will further help

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toward the designing of an immunocontraceptive vaccine for humans, based on synthetic peptide(s) corresponding to ZPB.

MATERIALS AND METHODS

Recombinant bmZPB

An internal cDNA fragment (1317 base pairs [bp]) corresponding to bmZPB excluding the N-terminal signal sequence and the C-terminus transmembrane-like domain was cloned in frame downstream of T5 promoter under the *lac* operator control in pQE-30 vector (pQE-ZPB, QIAexpress; Qiagen GmbH, Hilden, Germany) as described previously [12]. The r-bmZPB was expressed as polyhistidine (His_6) fusion protein in SG13009[pREP4] strain of *E. coli*. Transformed cells were grown at the shaker flask level (250 ml culture per flask; total volume 2 L) and induced with 1 mM isopropyl-β-D-thiogalactopyranoside when the cell density reached A_{600} of approximately 0.5–0.6. The r-bmZPB was purified using nickel-nitrilotriacetic acid resin [12]. The protein concentration was estimated using binchoninic acid (Sigma Chemical Co., St. Louis, MO).

Antibodies were raised in male New Zealand White rabbits against r-bmZPB as described previously [12]. The purified r-bmZPB was conjugated to diphtheria toxoid (DT) at 2:1 molar ratio also as described earlier [12].

Monoclonal Antibodies

Female BALB/c mice (Small Animal Facility, National Institute of Immunology, New Delhi, India) were immunized s.c. with r-bmZPB-DT conjugate (equivalent to 20 μg of r-bmZPB) in 0.9% saline emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, MI) and were boosted after 4 wk i.p. by the same amount of r-bmZPB-DT with incomplete Freund's adjuvant (Difco). After 8–10 wk, the mouse with the highest antibody titer against r-bmZPB (as determined by ELISA) was given i.v. injections (150 μl/injection) of r-bmZPB-DT (50 μg/day) through the tail vein for 3 consecutive days. The day after the booster injections, the animal was killed and the spleen removed aseptically. Splenocytes (9×10^6) were fused with SP2/O-Ag 1.4 mouse myeloma cells in a 2:1 ratio using 50% polyethylene glycol and were plated in six 24-well tissue culture plates. The hybrid cells were selected by growing the fused cells in RPMI-1640 medium supplemented with 20% fetal calf serum, 0.1 mM sodium hypoxanthine, 0.4 μM aminopterin, and 0.016 mM thymidine (Gibco BRL, Gaithersburg, MD). After 10 days of culture, the supernatants from all wells were checked for the production of antibodies against r-bmZPB in an ELISA. Cells from positive wells (positive for antibody against r-bmZPB in ELISA) were cloned by limited dilution to obtain stable hybrid cell clones secreting antibodies against r-bmZPB. The positive hybrid cells were grown as ascites in the peritoneal cavity of Pristane (Aldrich Chemical Co., Milwaukee, WI)-primed BALB/c mice. Immunoglobulins were purified by 40% ammonium sulfate precipitation followed by diethylaminoethyl A-50 (Pharmacia LKB, Uppsala, Sweden) column chromatography. The purified antibodies were concentrated using Amicon membrane (PM-30; Lexington, MA), dialyzed against PBS (50 mM phosphate, 150 mM NaCl, pH 7.4), and stored at −20°C. The purified immunoglobulins were quantitated spectrophotometrically at 280 nm.

Immunofluorescence on Bonnet Monkey Ovarian Sections and Human Oocytes

A normal cycling female bonnet monkey was ovariectomized, and the ovaries were snap frozen in liquid nitrogen. Ovarian sections of 5-μm thickness were cut in a cryostat at −20°C and fixed in chilled methanol. Sections passing through follicles were selected, washed with 50 mM PBS, pH 7.4, and blocked with 3% normal rabbit/goat serum at room temperature for 30 min. Sections were then washed 2 times with PBS and incubated at room temperature with respective mAbs (1:5 dilution of the culture supernatants) for 90 min. Negative controls included ovarian sections incubated with either PBS or culture medium obtained by growing SP2/O myeloma cells. Rabbit polyclonal antibodies (1:100 dilution) against r-bmZPB served as positive control. Sections were washed 3 times with PBS and incubated for 1 h at room temperature with 1:200 dilution of either rabbit anti-mouse immunoglobulin-fluorescein isothiocyanate (FITC) conjugate or goat anti-rabbit IgG-FITC conjugate (Dakopatts a/s, Glostrup, Denmark). Slides were washed with PBS, mounted in glycerol:PBS (9:1), and examined under a fluorescent microscope (Nikon, Tokyo, Japan).

The specific reactivity of mAbs with bonnet monkey ZP was further confirmed by inhibition of fluorescence in the presence of 12-mer bmZPB synthetic peptide (aa 136–147). Ovarian sections were incubated with respective purified mAbs from ascites (500 ng/ml) in the absence or presence of increasing concentrations of peptide, and slides were processed as described above. Synthetic peptide (aa 324–347) corresponding to bmZPC was used as a negative control.

Human oocytes that had failed to fertilize in standard in vitro fertilization-embryo transfer treatment because of severe male infertility factor were stored at −196°C in sperm-washing medium (SWM; Irvine Scientific, Santa Ana, CA) supplemented with 1.5 M propandiol, 0.1 M sucrose, and 10% cord serum after informed consent from donors was obtained. Before freezing, it was confirmed that all the oocytes had extruded the first polar body, but not the second one, suggesting that they were in metaphase II stage but not fertilized. Cryopreserved/thawed human oocytes were washed with PBS containing 0.1% BSA (PBS-BSA) to remove cryoprotectants. The oocytes were incubated with 1:200 dilution of either normal mouse serum or mAbs (ascites) at room temperature for 30 min. After washing with PBS-BSA, oocytes were further incubated at room temperature with goat anti-mouse IgG-FITC (Cappel, Turnhout, Belgium) at a dilution of 1:200 for 30 min. Subsequent to washing with PBS-BSA, the treated oocytes were mounted in glycerol:PBS (9:1) and examined under a fluorescent microscope.

Solid-Phase Peptide Synthesis

A panel of 12-mer overlapping peptides with 6 aa overlap corresponding to bmZPB precursor protein (aa 22–465), excluding the N-terminal signal sequence and C-terminus transmembrane-like domain, was synthesized. Peptides were synthesized in the carboxyl-to-amino-terminal direction using 9-fluorenylmethyloxycarbonyl-β-alanine group prederivatized polypropylene plastic pins as solid-phase support (Chiron Mimotopes Pty Ltd., Clayton, Australia) as described previously [14]. In addition, to map the minimum binding motif, overlapping octapeptides (shifting by one aa

at a time) were also synthesized. The pins were stored in the presence of desiccant until further use.

Peptides corresponding to bmZPB (aa 136–147) and bmZPC (aa 324–347) were synthesized on a Milligen 9050 synthesizer (Millipore, Bedford, MA) using fluorenylmethyloxycarbonyl chemistry. Peptides were side chain deprotected and cleaved using a mixture comprising trifluoroacetic acid (90%) with thioanisole (5%), ethane dithiol (3%), and anisole (2%) as the scavengers. The cleavage products were lyophilized and checked on a reversed-phase C-18 HPLC column.

ELISA

Reactivity of mAbs with r-bmZPB. Microtitration plates were coated with r-bmZPB (200 ng/well) in PBS at 37°C for 1 h and then at 4°C overnight. The plates were washed once with PBS and blocked with 1% BSA in PBS. All subsequent incubations were carried out at 37°C for 1 h. Each incubation was followed by washings done with PBS containing 0.05% Tween 20 (PBST). After blocking, the plates were incubated with either undiluted culture supernatant or purified mAbs at doubling dilutions. Bound antibodies were revealed with rabbit anti-mouse immunoglobulins conjugated to horseradish peroxidase at an optimized dilution of 1:3000 in PBST. Estimation of the enzyme activity was carried out with 0.05% orthophenylenediamine in 50 mM citrate phosphate buffer, pH 5.0, with 0.06% hydrogen peroxide as the substrate. The reaction was stopped with 50 μl of 5N H_2SO_4, and the absorbance was read at 492 nm with 620 nm as reference filter; values are expressed as antibody titers. Antibody titers were calculated by regression analysis and are expressed as antibody units (AU), i.e., reciprocal of the dilution of the ascites giving an absorbance of 1.0.

Inhibition assay. The inhibition in the binding of mAbs with r-bmZPB in the presence of 12-mer synthetic peptide (aa 136–147) corresponding to bmZPB was investigated by a competitive inhibition ELISA. Synthetic peptide (aa 324–347) corresponding to bmZPC was used as a negative control. The microtitration plates were coated with r-bmZPB as described above. Dilutions of mAbs giving an absorbance of 1.0 for binding to r-bmZPB in the absence of the competitor were determined. Subsequently, respective mAbs at 50% of the above dilution were incubated with an equal volume of increasing concentrations of competing peptide overnight at 4°C, and incubating mixtures were added in duplicate in 100 μl/well to r-bmZPB-precoated microtitration plates. After an incubation period of 2 h at 37°C, the plates were processed for the binding of mAbs as described above. A concentration of peptide resulting in 50% inhibition in the binding of mAb to r-bmZPB was determined by regression analysis and has been designated as IC_{50}.

Screening of multipin peptides with mAbs. Multipin peptides thus synthesized were checked for their specific binding to the mAbs by a modified ELISA as described previously [14]. Monoclonal antibodies purified from ascites were used at 1 and 10 μg/ml. Multipin peptides were reused by stripping off the bound antibodies from the pins by incubating them in disruption buffer (100 mM PBS, 1% SDS, 0.1% 2-mercaptoethanol, pH 7.2) at 65°C and sonicating for 10 min at 40-watt energy output using sonifier 450 (Branson Ultrasonic Corp., Danbury, CT). The pins were then rinsed with hot water, then by methanol, and air dried.

Sperm Binding and Penetration Assay

This assay and hemizona assay were performed according to the guidelines of the Japan Society of Obstetrics and Gynecology. In addition, all experiments using human oocytes and sperm were carried out under patient's informed consent. Human oocytes that had failed to fertilize in an in vitro fertilization-embryo transfer treatment because of severe male infertility factor at Hyogo College of Medicine, Japan, were examined carefully for sperm binding on the ZP and the stage of the oocytes before cryopreservation. Oocytes with germinal vesicle, denatured oocytes, and those oocytes penetrated by sperm that could not be removed by repeated vigorous pipetting were not used in these assays. Only those oocytes that had extruded the first polar body but not the second were selected for cryopreservation. Sperm were isolated from semen samples obtained from fertile healthy donors. After liquefaction, semen was centrifuged and washed twice in SWM containing 5.0 mg/ml of human serum albumin. The motile spermatozoa were collected by a standard swim-up technique and resuspended in SWM (2×10^7/ml). Cryopreserved oocytes were thawed and rinsed several times with Biggers-Whitten-Whittingham (BWW) medium supplemented with 4.0 mg/ml BSA (BWW-BSA medium) to remove cryoprotectants. The oocytes were treated with 10% (v:v) preimmune serum or 10% (v:v) immune serum for 1 h at 37°C in 5% CO_2 in air, followed by insemination at a concentration of 1×10^6 sperm/ml. After further incubation for 18 h, the oocytes were washed with fresh BWW-BSA medium in order to remove the spermatozoa loosely attached to the ZP with repeated pipetting. The total number of spermatozoa tightly bound to the ZP or the spermatozoa that penetrated into the ZP were counted under an inverted microscope. Statistical analysis was carried out by Student's *t*-test to compare differences between preimmune and immune sera.

Hemizona Assay

The hemizona assay was performed as described previously [15]. Frozen oocytes were thawed and rinsed in BWW-BSA medium and cut in half using micromanipulators (Narishige, Tokyo, Japan), mounted on a phase-contrast microscope (Nikon, Garden City, NY). One hemizona was placed in a 50-μl drop of either 1:10 or 1:100 dilution of ascites or purified immunoglobulins (1 mg/ml) of respective mAbs under mineral oil for 1 h at 37°C in 5% CO_2 in air, while the other matched hemizona was placed in a drop of normal mouse serum, 10% (v:v). In addition to normal mouse serum, in some experiments ascites fluid obtained by growing a mouse hybrid cell clone (P3W80) secreting mAb (IgG1) against hCG [16] was included as an additional negative control. After incubation, each hemizona was rinsed 5 times in fresh medium before exposure to 50 μl sperm suspension (2×10^6/ml). After 1 h of coincubation, each hemizona was removed and rinsed vigorously to detach loosely associated spermatozoa. The number of spermatozoa tightly bound to the outer hemizona surface were counted. The hemizona index (HZI) was calculated as follows: HZI = (number of bound spermatozoa in test serum / number of bound spermatozoa in control serum) × 100.

RESULTS

Polyclonal Rabbit Antibodies Against r-bmZPB Inhibited In Vitro Human Sperm-Egg Interaction

Generation of high antibody titers in male rabbits against r-bmZPB expressed in *Escherichia coli* was reported pre-

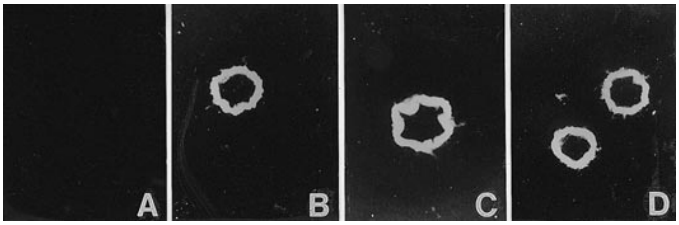

FIG. 1. Reactivity of mAbs with bonnet monkey ZP by indirect immunofluorescence: bonnet monkey ovarian cryosections (5 μm) were incubated with 1:5 dilution of the culture supernatant obtained by growing either SP2/O myeloma cells or hybrid cell clones and processed as described in *Materials and Methods.* Representative immunofluorescence patterns are shown: **A**) SP2/O culture supernatant, **B**) MA-801, **C**) MA-809, and **D**) MA-410 (×100; published at 52%).

TABLE 1. Effect of serum obtained from rabbit (R-137) immunized with r-bmZPB in human sperm binding and penetration assay.

Treatment[a]	No. of oocytes used	No. of spermatozoa bound/oocyte[b]	No. of spermatozoa penetrating/oocyte[b]
Experiment 1			
Preimmune serum	3	63.0 ± 9.8	4.0 ± 2.0
Immune serum	4	10.8 ± 6.4 ($P < 0.001$)	0.3 ± 0.5 ($P < 0.02$)
Experiment 2			
Preimmune serum	9	73.4 ± 29.9	6.7 ± 2.5
Immune serum	9	8.1 ± 5.9 ($P < 0.001$)	0.1 ± 0.3 ($P < 0.0001$)

[a] All serum used at 10% (v:v).
[b] Mean ± SD.

viously [12]. Initial characterization revealed that the anti-r-bmZPB antibodies recognized both bonnet monkey and human ZP as tested in an indirect immunofluorescence assay [12,17]. In order to assess the contraceptive potential of the antibodies raised against r-bmZPB, we employed an in vitro human sperm binding and penetration assay. The immune serum from rabbit (R-137) immunized with r-bmZPB as compared to preimmune serum showed a significant inhibition in the binding of spermatozoa to antibody-treated oocytes (Table 1). A similar effect was observed in two independent experiments. In addition to a decrease in the number of sperm bound to ZP in the presence of immune serum, a decrease in the number of sperm penetrating the ZP was also observed (Table 1). No tangible effect on either the motility or auto-agglutination of the sperm was observed after incubation in the presence of immune serum as compared to preimmune serum (data not shown).

Monoclonal Antibodies Against r-bmZPB

To further characterize the motifs on ZPB that when complexed with respective antibodies will inhibit sperm-oocyte binding, a panel of 7 mAbs was generated against r-bmZPB. In addition, mAb MA-410, previously generated against porcine ZPB (a homologue of bmZPB) and cross-reactive with bonnet monkey ZP, was included for evaluation. The characteristics of these mAbs are summarized in Table 2. MA-801, -811, -813, -820, -825, and -410 were of the IgG1 isotype; MA-809 was IgG2a isotype, and MA-824 was IgA isotype. All the mAbs showed high reactivity (antibody titer $> 10^5$ AU) with r-bmZPB in the ELISA.

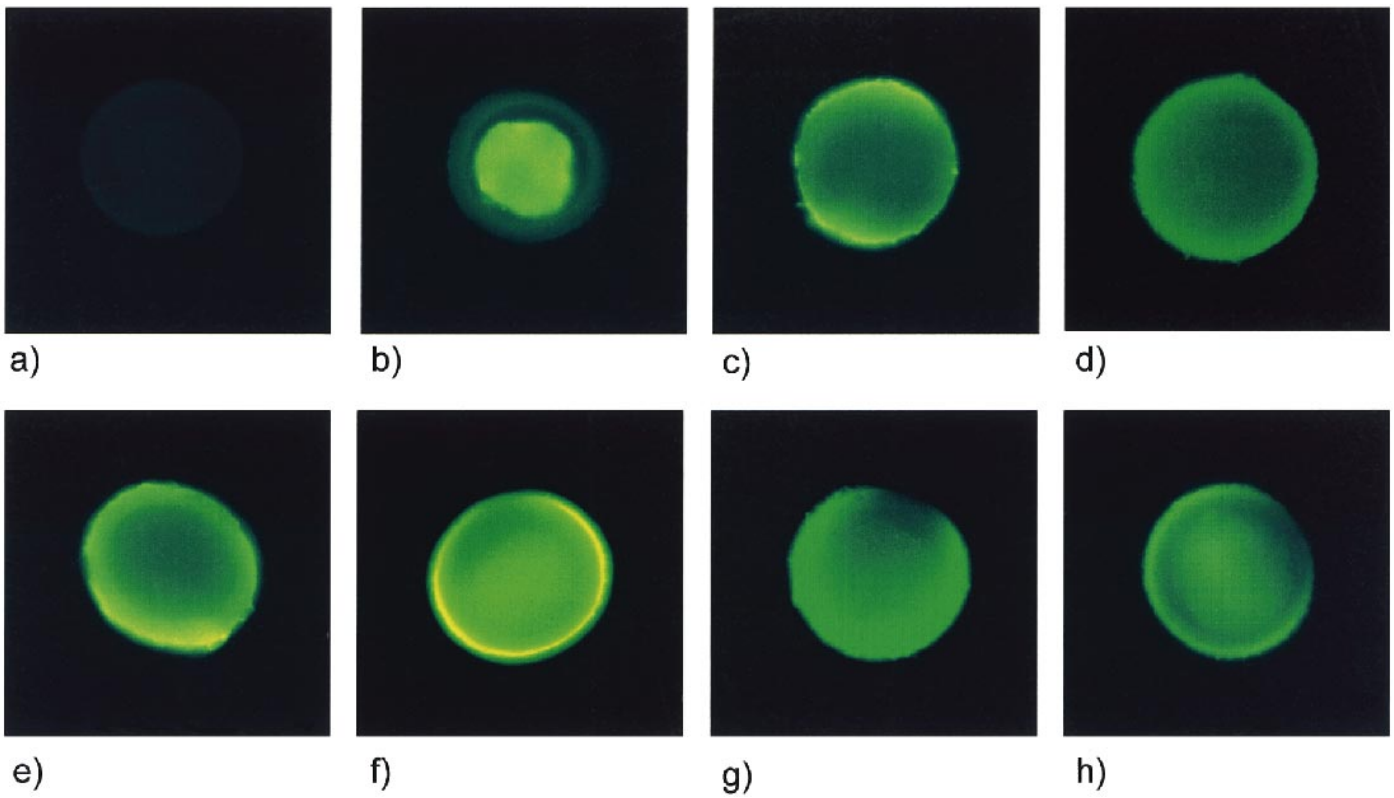

FIG. 2. Reactivity of mAbs with human oocytes by indirect immunofluorescence: oocytes were incubated with 1:200 dilution of the mAbs (ascites) or normal mouse serum followed by goat anti-mouse IgG-FITC conjugate. Representative immunofluorescence patterns are shown: **a**) normal mouse serum, **b**) MA-801, **c**) MA-809, **d**) MA-811, **e**) MA-813, **f**) MA-820, **g**) MA-825, and **h**) MA-410 (×100; published at 72%).

TABLE 2. Characteristics of monoclonal antibodies generated against r-bmZPB and porcine ZPB.

mAb	Immunogen	Isotype	ELISA antibody titer[a,b] (AU; $\times 10^5$)	Immunofluorescence[c] with Human oocyte	Immunofluorescence[c] with Bonnet ZP
MA-801	r-bmZPB	IgG1	4.04 (37.6)	+	+
MA-809	r-bmZPB	IgG2a	7.59 (32.5)	+	+
MA-811	r-bmZPB	IgG1	4.90 (25.5)	+	+
MA-813	r-bmZPB	IgG1	3.90 (23.4)	+	+
MA-820	r-bmZPB	IgG1	1.60 (18.3)	+	+
MA-824	r-bmZPB	IgA	1.72 (27.5)	−	+
MA-825	r-bmZPB	IgG1	4.55 (32.1)	+	+
MA-410	porcine ZPB	IgG1	1.06 (29.5)	+	+

[a] AU, antibody units, i.e., ascites dilution giving an A_{492} of 1.0.
[b] Values in parentheses represent the concentration of purified immunoglobulins in the ascites (mg/ml).
[c] +, Positive; −, negative.

The ability of mAbs to recognize native ZP was evaluated by an indirect immunofluorescence assay using cryosections of bonnet monkey ovary and human oocytes. All 8 mAbs reacted with bonnet monkey ZP. The typical reactivity pattern of MA-809, -813, and -410 with bonnet ZP is shown in Figure 1. All 3 mAbs showed intense fluorescence with the ZP and did not react with any other ovarian cell types. The culture supernatant obtained by growing SP2/O myeloma cells failed to show any fluorescence (Fig. 1). Of the 8 mAbs, 7 showed positive though variable reactivity with human oocytes (Fig. 2). MA-809 and MA-813 showed more intense fluorescence with the outer rim of the ZP, whereas MA-820 showed more intense fluorescence with the inner rim of the ZP. MA-811, -825, and -410 showed a uniform fluorescence with ZP of human oocytes. Comparatively, MA-801 showed weak fluorescence with ZP but an intense fluorescence with the cytoplasm of the oocyte. Normal mouse serum (Fig. 2), MA-824, and P3W80 (data not shown) failed to show any positive fluorescence.

Monoclonal Antibodies Against r-bmZPB Inhibited Binding of Human Spermatozoa to Human Zona in a Hemizona Assay

The contraceptive potential of mAbs was evaluated in a hemizona assay, and the results are summarized in Table 3. No significant inhibition in the HZI was observed with MA-801, -820, -824, and -410 when used as ascites at 1:10 dilution. At similar dilution of ascites MA-809, -811, -813, and -825 showed an HZI of 26.4, 36.2, 20.5, and 31.1, respectively, which is significant with regard to the criteria described earlier [18,19]. A significant inhibition in the HZI (29.1) was also observed at higher dilution (1:100) of MA-813. Purified MA-813 and MA-825 (1 mg/ml) also showed a significant inhibition in the HZI (Table 3).

Monoclonal Antibodies Having In Vitro Contraceptive Potential Recognized a Motif Conserved in Bonnet Monkey and hZPB

To map the epitopes recognized by mAbs, dodecapeptides with a 6 aa overlap corresponding to the bmZPB deduced aa sequence were synthesized using the multipin peptide synthesis approach. The purified mAbs were tested at 1 and 10 μg/ml for binding to the peptides in a direct binding enzyme immunoassay. The binding motif for MA-809, -811, -813, and -825 was mapped to DAPDTDWCDSIP (aa 136 to 147; numbering of aa is based on the bmZPB precursor sequence) (Fig. 3). MA-801, -820, and -824 when tested at 10 μg/ml showed reactivity with multiple pins but at 1 μg/ml (equivalent concentration as compared to other tested mAbs) failed to show any discrete binding with any peptide (data not shown). MA-410 was mapped to aa residues 250–261 (GDRAVYENELVA) corresponding to the precursor bmZPB protein (Fig. 3). Monoclonal antibody P3W80 having specificity for hCG failed to show reactivity with any peptide (data not shown). Interestingly, the motif recognized by MA-809, -811, -813, and -825 on bmZPB was completely conserved with hZPB (Table 4). To further localize the minimum binding motif recognized by MA-809, -811, -813, and -825, overlapping octapeptides (shifting by one aa at a time) were synthesized and screened in ELISA. The minimal binding motif for MA-809, -811, -813, and -825 was mapped to DWC, and typical results obtained with MA-813 are shown in Figure 4.

TABLE 3. Effect of mAbs against r-bmZPB/porcine ZPB on binding of human sperm to human zona in a hemizona assay.

MAb	No. bound sperm/hemizona: Control group	No. bound sperm/hemizona: Antibody treated group	HZI
MA-801	110	102	98.7
(ascites 1:10)	89	82	
	40	52	
MA-809	67	40	26.4
(ascites 1:10)	128	33	
	65	8	
	73	7	
MA-811	49[b]	19	36.2
(ascites 1:10)	40[b]	8	
	99[b]	41	
MA-813	72	16	20.5
(ascites 1:10)	61	3	
	33	15	
MA-813	102[b]	23	29.1
(ascites 1:100)	67[b]	21	
	44[b]	18	
MA-813	55[b]	6	19.0
(purified Ig 1 mg/ml)	94[b]	17	
	46[b]	14	
MA-820	96	54	58.1
(ascites 1:10)	38	18	
	81	53	
MA-824	98	48	101.3
(ascites 1:10)	61	113	
MA-825	45[b]	12	31.1
(ascites 1:10)	38[b]	9	
	52[b]	21	
MA-825	66[b]	19	35.4
(purified Ig 1 mg/ml)	49[b]	32	
	60[b]	11	
MA-410[a]	107	90	73.1
(ascites 1:10)	75	32	
	38	39	

[a] mAb against porcine ZPB.
[b] P3W80 ascites (mAb against hCG) used at 1:10 dilution instead of normal mouse serum (10% v:v).

Synthetic Peptide Corresponding to Mapped Epitope Inhibited Binding of mAbs to r-bmZPB and Native ZP

To substantiate the epitope-mapping data, bmZPB synthetic peptide corresponding to the mapped region (aa 136–

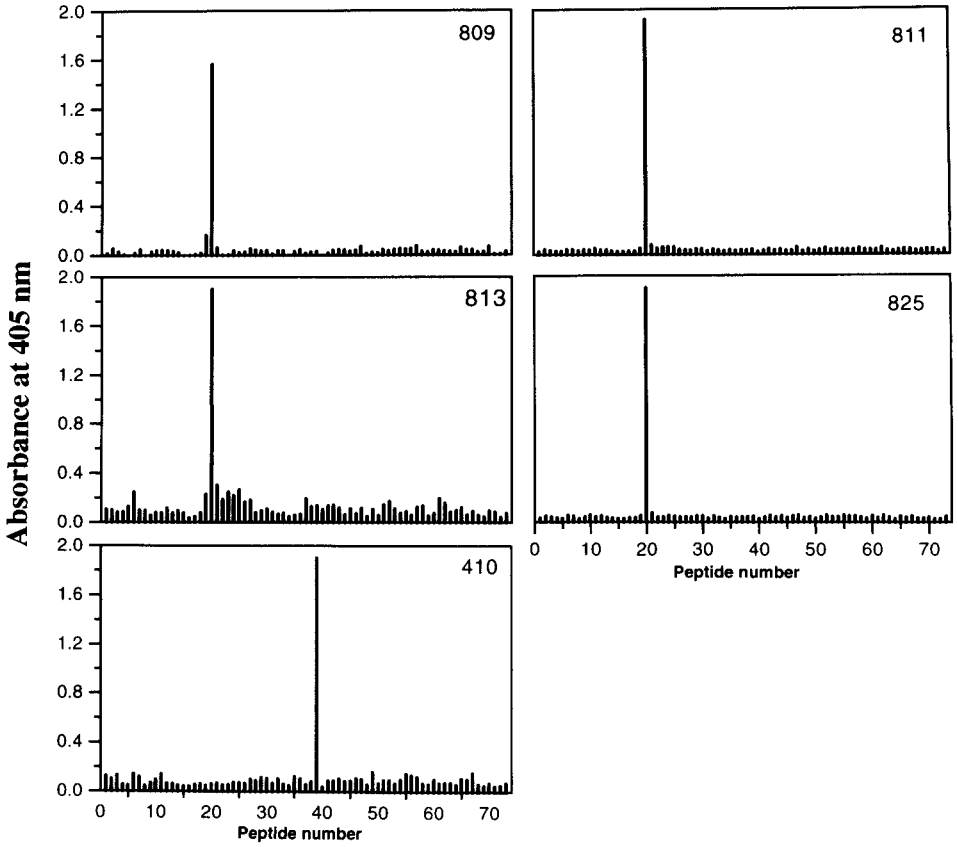

FIG. 3. Determination of binding motifs of mAbs on bmZPB: overlapping 12-mer peptides with an overlap of 6 aa, corresponding to aa residues 22–465 of bmZPB (aa numbering based on the deduced aa sequence of precursor bmZPB cDNA), were synthesized on polypropylene pins (represented on x-axis) and screened for the reactivity with mAbs (1 μg/ml) in an ELISA (see *Materials and Methods*). Values expressed as absorbance were obtained after subtracting absorbance obtained for the negative control peptide (GLAQ; Chiron) supplied by the manufacturer from the absorbance obtained for the given peptide by respective antibodies.

147) was made. Purified mAbs MA-809, -811, -813, and -825 when tested at 1 μg/ml (100 ng/well) showed specific reactivity with synthetic peptide (microtitration plate coated with 1 μg peptide per well) in an ELISA (Table 5). MA-801, -820, -824, and -410 failed to react with the bmZPB peptide. Further, inhibition in the binding of the respective mAbs to r-bmZPB in the presence of increasing concentration of the bmZPB synthetic peptide was assessed. Synthetic peptide showed dose-dependent inhibition in the binding of MA-809, -811, -813, and -825 to r-bmZPB. The IC_{50} values of the competing peptide with respect to various mAbs are shown in Table 5. No significant inhibition in the binding of MA-801, -820, -824, and -410 was observed in the presence of competing peptide when tested up to 749.10 pM. The inhibition in the binding of MA-809, -811, -813, and -825 to r-bmZPB was specific, as synthetic peptide corresponding to bmZPC (aa 324–347) when tested up to 401.76 pM failed to inhibit their binding.

Purified mAbs against r-bmZPB when tested at 500 ng/ml showed intense fluorescence with bonnet monkey ZP in an indirect immunofluorescence assay. A typical reactivity pattern with MA-813 is shown in Figure 5A. Prior incubation of mAbs MA-809, -811, -813, and -825 with increasing concentrations of bmZPB peptide resulted in partial inhibition of binding of respective mAbs to bonnet monkey ZP at 1 μg/ml of peptide and complete inhibition at and above 10 μg/ml of peptide. Inhibition in the fluorescence by MA-813 in the presence of bmZPB peptide is shown in Figure 5B. However, no inhibition was observed in the presence of bmZPC synthetic peptide up to 100 μg/ml (data not shown). Binding of MA-801, -820, -824, and -410 to bonnet monkey ZP was not influenced by the presence of bmZPB or bmZPC peptides when evaluated up to 100 μg/ml (data not shown).

DISCUSSION

ZP glycoproteins, by virtue of their critical involvement in fertilization, have been proposed as candidate antigens for design of an immunocontraceptive vaccine [20]. Their use as immunogen for fertility regulation in humans has been hampered by the observations that although active im-

TABLE 4. Alignment of the B cell epitopic sequences recognized by the mAbs against bmZPB having contraceptive efficacy with its analogues from different species.[a]

Species	Epitopic sequences															
Bonnet					D	A	P	D	T	—	—	D	W	C	D	S	I	P
Human					*	*	*	*	*	—	—	*	*	*	*	*	*	*
Rabbit					P	*	*	*	A	—	—	G	L	*	*	*	V	*
Cat					E	*	*	N	A	—	—	*	L	*	*	*	V	*
Pig	**L**	**L**	**A**	**T**	*	**V**	*	**T**	**I**	—	—	**G**	**L**	*	*	A	V	*
Mouse					V	G	T	H	L	P	Q	E	R	*	Q	V	A	S

[a] The epitope represented in bold and underlined is recognized by MA-420 generated against porcine ZPB [27].
* Asterisks indicate completely conserved aa.

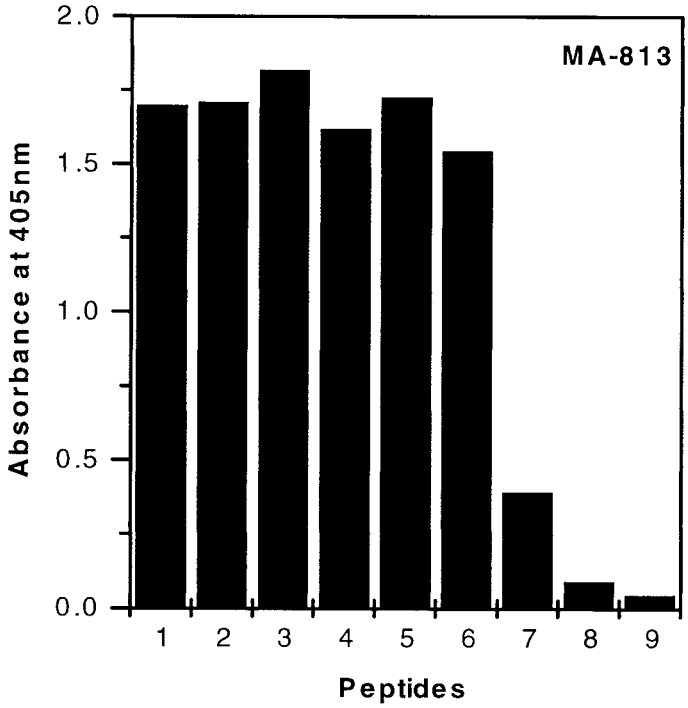

FIG. 4. Determination of minimum binding motif for mAb: overlapping octapeptides corresponding to aa residues 136–151 of bmZPB precursor protein were synthesized on polypropylene pins (P1: DAPDTDWC, P2: APDTDWCD, P3: PDTDWCDS, P4: DTDWCDSI, P5: TDWCDSIP, P6: DWCDSIPA, P7: WCDSIPAR, P8: CDSIPARD, P9: DSIPARDR) and screened for reactivity with MA-813 by ELISA. Other details are the same as in legend to Figure 3.

TABLE 5. Reactivity in ELISA of mAbs with bmZPB synthetic peptide corresponding to mapped epitope.

mAb	A_{492}[a]	Competitor IC_{50} (pM)[b]	
		bmZPB peptide[c]	bmZPC peptide[d]
MA-801	NB	NS	NSI
MA-809	2.81	07.44 ± 0.49	NSI
MA-811	2.34	17.50 ± 0.26	NSI
MA-813	2.36	19.40 ± 0.25	NSI
MA-820	NB	NS	NSI
MA-824	NB	NS	NSI
MA-825	2.76	07.92 ± 0.05	NSI
MA-410	NB	NS	NSI

[a] Reactivity of purified mAbs (1 μg/ml; 100 ng/well) with bmZPB synthetic peptide (aa 136–147) in ELISA using microtitration plate coated with 1 μg peptide per well; results are expressed as the arithmetic mean of duplicates. NB represents no significant binding with the peptide.
[b] The dose-dependent regression curves were used to calculate IC_{50} values for bmZPB synthetic peptide. Values represent mean ± SEM for three independent experiments.
[c] NS denotes no significant inhibition in the binding of the mAb to r-bmZPB in the presence of bmZPB synthetic peptide when tested up to 749.10 pM.
[d] NSI denotes no significant inhibition in the binding of the mAb to r-bmZPB in the presence of bmZPC synthetic peptide when tested up to 401.76 pM.

munization with various ZP glycoproteins/proteins leads to block in fertility, this block of fertility is not entirely at the level of sperm-oocyte interaction but is also due to alteration in follicular development. Among the various factors to which these observations are attributed are 1) possible contamination of the purified zona glycoproteins with other ovarian glycoproteins and 2) the presence of oophoritogenic T cell epitopes in zona-based immunogens. Lou et al. [11] succeeded in segregating an oophoritogenic T cell epitope of mouse ZPC from a B cell epitope, capable of eliciting an immune response leading to block of fertility without ovarian pathology. Attempts have also been made to test the efficacy of antibodies generated against synthetic peptides corresponding to mouse and human ZPA and ZPC to inhibit in vitro human sperm-oocyte binding [21,22]. Antibodies raised against synthetic peptides corresponding to ZPA and ZPC recognized the native zonae. However, antibodies against ZPA peptide (aa residues 541–555) alone inhibited in vitro human sperm-oocyte binding [21].

Availability of r-bmZPB expressed in *E. coli*, which is devoid of other ovarian proteins as contaminant, prompted us to delineate the B cell epitope relevant for design of future immunocontraceptive vaccines based on synthetic peptides. Significant inhibition in the binding of human sperm to the antibody-treated zona-encased human oocyte in vitro by the polyclonal antibodies against r-bmZPB is an interesting though not surprising observation. Polyclonal antibodies against porcine ZPB showed a significant inhibition in the binding of boar sperm to antibody-treated zona-encased oocytes [23]. Antisera raised in rabbits and marmosets to porcine ZP and/or deglycosylated ZP also inhibited the binding of human sperm to zona-encased human oocytes [24]. Efficacy of antibodies in a heterologous assay system to inhibit sperm-oocyte interaction could be due to a variable degree of interspecies aa sequence identity of the ZP glycoprotein. High aa sequence identity (92.0%) between bmZPB and hZPB may explain the efficacy of the antibodies against r-bmZPB to inhibit the human sperm-oocyte interaction in vitro [12]. However, denatured recombinant proteins as compared to native glycoproteins/proteins may not necessarily generate an appropriate antibody repertoire, which may interfere with its desired biological function. Polyclonal antibodies raised in rabbits against r-bmZPC (94% aa sequence identity between bmZPC and hZPC) expressed in *E. coli* failed to inhibit the binding of human sperm to antibody-treated zona-encased oocytes (unpublished results). However, rabbit polyclonal antibodies generated against synthetic peptides corresponding to aa 324–347 (KGDCGTPSHSRRQPHVVSQWSRSA) of bm-

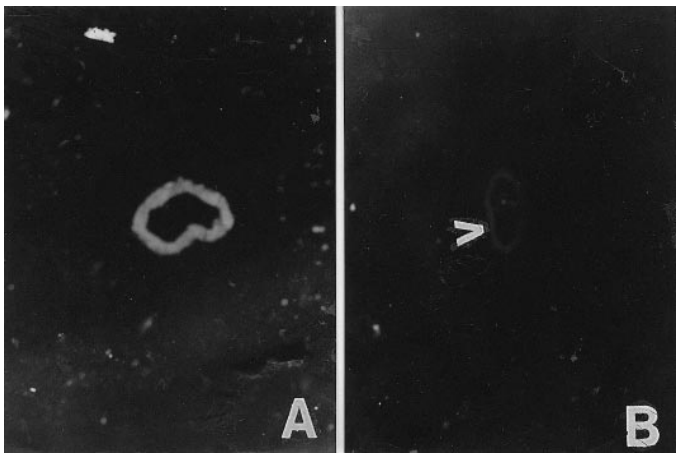

FIG. 5. Reactivity of mAbs in presence of bmZPB synthetic peptide with bonnet monkey ZP by indirect immunofluorescence: bonnet monkey ovarian cryosections were incubated with purified mAbs (500 ng/ml) in either absence or presence of increasing concentrations of bmZPB peptide. Representative immunofluorescence pattern are shown: **A**) MA-813; **B**) MA-813 + 10 μg/ml of bmZPB synthetic peptide (aa 136–147) (×100; published at 88%). Arrowhead indicates inhibition in immunofluorescence of ZP.

ZPC and coupled to DT significantly inhibited in vitro human sperm-oocyte binding [25].

In order to map the immunologically relevant B cell epitopes of bmZPB, murine mAbs against r-bmZPB have been generated. Hybrid cell clones grown as ascites showed high mAb titers ranging from 1.60×10^5 (MA-820) to 7.59×10^5 (MA-809) antibody units as tested in an ELISA (Table 2). All 7 mAbs reacted to bonnet monkey ZP, and 6 recognized human ZP. The failure of reactivity of the MA-824 with human ZP may be due to the fact that it is an IgA isotype and therefore anti-mouse IgG-FITC conjugate employed in this experiment could not recognize zona-bound MA-824. Monoclonal antibodies MA-809 and MA-813, showing an intense immunofluorescence on the outer surface of ZP, were effective in inhibiting in vitro the binding of human sperm to human zona in a hemizona assay. However, intense immunofluorescence by mAbs on the outer surface of the zona may not be an important criterion, as mAbs MA-811 and MA-825, showing more or less uniform immunofluorescence on the zona, also showed significant inhibition in the hemizona assay. More intense immunofluorescence by MA-801 with the cytoplasm as compared to the ZP of the human oocyte may suggest that this mAb recognizes ZPB, which may not have been incorporated in the zona matrix.

In vitro inhibition in the binding of human sperm to human zona in a hemizona assay by mAbs MA-809, -811, -813, and -825 is specific, as MA-801, -824, and -410 failed to show inhibition in spite of having comparable antibody titers (Tables 2 and 3). MA-813 showed significant inhibition in the hemizona assay even when tested at one log-higher dilution. Nonspecific inhibition by ascites is further ruled out by the observations that purified MA-813 and MA-825 also bring about a significant inhibition in the binding of sperm to zona. Further, inhibition in binding of the sperm to zona by MA-811, -813, and -825 was observed with respect to the matched hemizona incubated with P3W80 ascites at the same (MA-811 and MA-825) or lower (MA-813) dilution (Table 3). Previous studies from our laboratory suggested that in the porcine model system, the inhibition of sperm-egg interaction by mAbs is the function of neither the affinity nor the isotype of the mAbs employed [26]. However, the epitopic domains recognized by the respective mAbs were probably more relevant in interfering with gamete interaction. It appears likely that MA-809, -811, -813, and -825, within the limitations of the assay conditions, may be either reacting directly with the binding domain on the zona that is the putative sperm receptor or reacting with a region close to the receptor site and bringing about the inhibition in binding of sperm by steric hindrance.

Synthetic 12-mer peptides with 6 aa overlap corresponding to bmZPB deduced aa sequence, excluding the signal sequence and transmembrane-like domain, were screened in direct binding ELISA to map the epitopes recognized by bioeffective mAbs. MA-809, -811, -813, and -825 recognized epitope corresponding to aa 136 to 147. Dose-dependent inhibition in the binding of these mAbs by bmZPB synthetic peptide corresponding to the mapped epitope (aa 136–147), and not by bmZPC peptide (aa 324–347) to r-bmZPB in an ELISA and native bonnet monkey ZPB in an indirect immunofluorescence assay, further confirmed this observation. Further defining of the epitope by making use of octamer peptides revealed that the minimum binding motif of these was from aa 141 to 143. Reactivity of mAbs MA-801, -820, and -824 when tested at 10 μg/ml with multiple but discrete peptides of bmZPB may represent nonspecific binding, as these antibodies failed to show any specific binding to a particular peptide at a lower concentration of 1 μg/ml. Hence the methodology used by us failed to map the epitopes recognized by these mAbs. Previous epitope-mapping studies using mAbs against porcine ZPB, purified from pig ovaries, revealed that one of the mAbs, MA-420, capable of inhibiting porcine gamete interaction, recognizes residues corresponding to aa 133–144 (numbering of the aa is based on the precursor porcine ZPB deduced aa sequence) [27]. Interestingly, this epitopic domain overlaps with the epitope recognized by MA-809, -811, -813, and -825. Within the B cell epitope mapped by MA-420 as compared to MA-809, -811, -813, and -825, of the 8 overlapping aa residues, only 3 aa are identical (Table 4). The mapped B cell epitope on bmZPB revealed that of 12 aa, 7 aa are identical with the analogous epitope of rabbit and cat ZPB, 5 aa in the case of pig ZPB, and just 1 aa in the case of mouse ZPB (Table 4). It may be of interest to note that the epitope mapped by Millar et al. [28] on mouse ZPC by a bioeffective mAb also lies in a region that is less conserved in various mammalian species. B cell epitope-mapping studies by our group using mAbs generated against porcine ZPB and ZPC and capable of interfering in pig sperm-oocyte interaction, revealed that the motifs recognized by these antibodies had variable degrees of aa sequence identity with their analogous epitope from different species [27,29]. Interestingly, the epitope recognized by bioeffective mAbs MA-809, -811, -813, and -825 is fully conserved in bonnet monkey as well as hZPB.

To our knowledge these studies have delineated for the first time the epitope of nonhuman primate ZPB, which has relevance for designing a synthetic peptide-based immunogen for fertility regulation in humans. However, it remains to be ascertained whether a synthetic peptide encompassing this motif will generate antibody response capable of interfering with gamete interaction but not generating T cell response.

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